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Genetics of alkane degradation in *Pseudomonas oleovorans*

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SUMMARY AND GENERAL CONCLUSIONS

The catabolic plasmid OCT extends the substrate range of *P. putida* from alkanols to n-alkanes and n-alkenes with a chain length between C₆ and C₁₂. The alk regulon, that is localized on this plasmid, consists of two distinct regions, the alkBAC operon and the alkR locus which positively controls the expression of the alkBAC operon. The alkBAC operon encodes for two enzymatic functions: alkane hydroxylation and oxidation of the resulting alkanol. The oxidation of aliphatic compounds by *P. oleovorans* (*P. putida* carrying the OCT plasmid) is of interest due to the specific characteristics of this organism which grows in the presence of a bulk apolar phase and due to the potential utility of terminal oxidation in the production of alkanols, aldehydes, dicarboxylic acids, chiral epoxides and polyesters.

The research described in this thesis was aimed at a detailed genetic characterization of the genes responsible for alkane oxidation. Since the large 400-500 kb OCT plasmid which carries the alk genes cannot be handled for *in vitro* characterization and manipulation, a gene library was established with total *P. oleovorans* DNA. For this purpose the broad host range binary cosmid plasmid system pLAFRI/pRK2013 (Friedman et al., 1982) was used, which appeared to function very well, both in terms of genetic stability and mobilization from *E. coli* towards *P. putida*. The alkBAC operon, carrying the structural genes, and the alkR locus, carrying the regulatory functions, were identified by introducing the gene library into *P. putida* strains carrying alk mutations (Chapter 2).

The complete alkBAC operon was located on a 16.9 kb *EcoRI* fragment, which complemented all structural alk mutations. Although in the absence of the alkR locus the cloned alkBAC operon is not expressed in *P. putida*, one spontaneous Alk⁺ mutant could be isolated, suggesting that all structural OCT derived alk genes are present on this fragment. The size and structure of the alkBAC operon was determined by R-looping experiments and analysis for protein content in *E. coli* minicells (Chapter 3). The length of the operon is approximately 7.5 kb and six polypeptides were found to be encoded by this operon: 41, 15, 49, 58, 59 and 20 kDa, respectively (Fig. 1). The 41 kDa peptide (alkB) was shown to be alkane hydroxylase in immunoprecipitation experiments. Sequence

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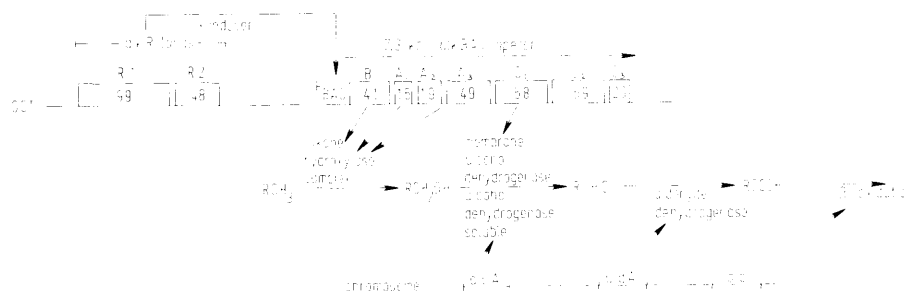


Fig. 1. Scheme of genes and gene products involved in alkane oxidation in *Pseudomonas oleovorans*.

data of the first half of the operon (M. Kok, to be published) have confirmed the identity and the location of the alkB cistron and revealed the existence of two rubredoxin cistrons encoding a 15 and 19 kDa polypeptide, respectively. Although it is likely to assume that the 49 kDa protein is rubredoxin reductase, no direct genetic evidence has been obtained and recent analysis of the DNA sequence revealed homology with human aldehyde dehydrogenase (M. Kok and P. Terpstra, unpublished results). Mapping of alkC mutations has shown that the 58 kDa protein is involved in alkanol dehydrogenase activity (Owen et al., 1984; Chapter 2 and 4). It remains to be determined whether the 59 kDa protein is also a component of the alkanol dehydrogenase.

The alkR locus was found to reside on an 18 kb EcoRI fragment, which could be further subcloned to a 4.9 kb SalI fragment. With the availability of the cloned structural and regulatory alk sequences, we were able to reconstitute the alk system by coinserting the two EcoRI fragments in a the broad host range vector pLAFRI (Chapter 4). It appeared that pLAFRI containing the complete alk system endowed *P. putida* with a wild type Alk phenotype, which implies that no other functions encoded by the OCT plasmid are required for alkane utilization. Furthermore we obtained recombinant plasmids in which the last three cistrons were deleted, yielding an alkBA/R plasmid. These constructs were introduced into several *P. putida* and *P. oleovorans* hosts, and tested for their production characteristics in multi phase bioreactors (R.G. Lageveen, 1987). The initial results for the conversion of octane to octanol are very promising since maximal

conversion rates of 50 U/g were reached.

Interestingly, we found that *E. coli* equipped with either alkBAC/R or alkBA/R is able to grow on octane, provided that the fatty acid degradation genes are expressed constitutively. Whereas the fatty acid degradation enzymes oxidize both long and medium chain fatty acids, the expression of the fad genes, which is under negative control of a 29 kDa protein (fadR), can only be induced by long chain fatty acids (DiRusso and Nunn, 1985). This illustrates once more that the substrate range of metabolic routes in microorganisms is also a reflection of the inducer specificity of regulatory proteins. The same phenomenon was observed for the alk system of which undecane and dodecanol are non-inducing substrates (Fennewald and Shapiro, 1977).

The expression of the alk genes was studied using specific antibodies raised against the first gene product of the alkBAC operon, alkane hydroxylase. The induction kinetics is similar in *E. coli* and *Pseudomonas*, and the high levels of protein reached reveal that the alk system is expressed efficiently. After two hours of induction a steady state level is reached in which time the alkane hydroxylase accounts for about 1.5% of total newly synthesized protein, which corresponds with about 35,000 copies of alkane hydroxylase per cell. Generally *Pseudomonas* genes are poorly expressed in *E. coli*, which is ascribed to inefficient transcription initiation of *E. coli* RNA polymerases (Jeenes et al., 1986). However, the alk system is the first *Pseudomonas* derived system which is expressed identically in *Pseudomonas* and *E. coli*, and is therefore an interesting candidate for a broad host range expression system.

The development of such a expression system requires a detailed analysis of the regulatory region of the alkBAC operon (M. Kok, to be published) and an adequate understanding of the genetic organization of the alkR locus. From analysis in *E. coli* minicells we determined that the alkR locus codes for a 99 kDa and a 48 kDa peptide (Chapter 5). We have established the positions of the corresponding encoding cistrons, which can be expressed independently of one another and which were shown to be both essential for expression of the alkBAC operon. AlkR mutations which can revert to an Alk^+ phenotype with an altered inducer specificity were mapped in the alkR1 cistron coding for the 99 kDa protein. Combined with the suggestion of Benson (1979) who found indications that a

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membrane bound component is involved in regulation of alkBAC expression, we propose a two component regulatory system. The inducer is recognized by the membrane bound 99 kDa protein which transfers an inducer recognition signal towards the soluble component, the 48 kDa polypeptide. This peptide then activates transcription of the alkBAC operon by interacting with either the regulatory region or the RNA polymerase.

Although the mechanism of control of alkBAC expression is not yet clear, it is obvious that we have uncovered an intriguing regulatory system. The availability of the cloned alkR cistrons, and the variety of alkR mutants (Fennewald and Shapiro, 1977), including constitutive mutants, should enable us to proceed with a detailed study on the specific role and cellular localization of each of the regulatory components.

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